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# Regulation of Alcohol Dehydrogenases in Maize Scutellum during Germination[1]

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## ABSTRACT

**The pattern of change in the activity of alcohol dehydrogenase in maize (*Zea mays* L.) scutellum during seed germination is not altered by 10 μg/ml cycloheximide or 50 μg/ml actinomycin D. The enzyme does not become density labeled when maize seeds are germinated in the presence of $D_2O$ and $^{15}NH_4Cl$, indicating that no new alcohol dehydrogenase molecules are synthesized after the onset of germination. However, the activity of an endogenous inhibitor for alcohol dehydrogenase is increased after germination. The increase of this inhibitor is concomitant with the decline of alcohol dehydrogenase activity, indicating that the activity of alcohol dehydrogenase during seed germination is controlled by the level of the inhibitor.**

A change in the activity of various enzymes is commonly observed during and after the onset of germination. The activity of those enzymes characteristic of maturing seeds and those required for metabolism in the dry seed will be decreased, while enzymes needed for the germination process will be increased in activity.

Alcohol dehydrogenase (alcohol:NAD oxidoreductase, EC 1.1.1.1; abbreviated as ADH) appears as four distinct zones of activity (ADH-1, ADH-2, ADH-3, and ADH-4) on zymograms of scutellar extracts from mature kernels (10). The genetic basis for some of these isozymes has been determined (11). Physiologically, ADH in plant tissues is probably necessary for anaerobic glycolysis which may be of importance in the metabolism of the resting seed. However, after seed germination the increase in aerobic respiration may obviate the need for ADH.

In order to study the control mechanism(s) regulating the activity of alcohol dehydrogenase during and after germination, we have employed a density labeling technique, which is an effective means for detecting the *de novo* synthesis of protein molecules (1, 4, 8), and have found that there is no *de novo* synthesis of ADH molecules during this developmental period. We also found that the level of a specific inhibitor for ADH increases during maize seed germination. These facts lead us to suggest that the structural genes for ADH are probably repressed in the scutellum by some mechanism before or during the onset of the germination process and that the decline of ADH activity is due to the increase of the ADH specific inhibitor.

## MATERIALS AND METHODS

**Preparation of Seeds.** Maize (*Zea mays* L.) seeds, inbred line W64A, were surface-sterilized with 5% sodium hypochlorite for 10 min, washed with sterilized deionized $H_2O$, and germinated between moistened germination paper (Kimpak) in the dark at 24 C. In the density labeling experiments, the germination paper was replaced by two pieces of Whatman No. 1 filter paper moistened either with 10 mM $^{14}NH_4Cl$ in $H_2O$ as control, or with 10 mM $^{15}NH_4Cl$ in 70% $D_2O$. For the drug inhibitor experiments, the scutella were excised under sterile conditions after 24 hr of germination and placed on filter paper moistened with Hoagland's solution. Extracts were prepared by homogenizing the scutella with sand in a mortar and pestle containing 25 mM glycylglycine buffer (pH 7.4) with 0.1 mM β-mercaptoethanol, and centrifuging at 20,000*g* in a Sorvall RC-2B for 10 min. The supernatant was used in all assays.

**Enzyme Assay.** Alcohol dehydrogenase activity was measured essentially as described by Scandalios and Felder (10); the absorbance of the reduced form of NAD was recorded at 340 nm on a Gilford Model 2400 recording spectrophotometer. Lactate dehydrogenase activity was determined according to the method of Kornberg (5).

**Assay of ADH Inhibitor.** The extract to be assayed for inhibitor was mixed with an equal volume of scutellar extract from 2-hr imbibed seeds. The scutellar extract and the inhibitor extract were mixed separately with an equal volume of buffer. After incubation at 37 C for 1 to 3 hr, the sum of ADH activity in the two extract-buffer mixtures was designated as control (100%). The residual activity in the extract-extract mixture was also assayed. The content of inhibitor was expressed as percentage ADH activity lost with respect to the control. Purified ADH was occasionally used in this assay, and the results were similar to those assays done with scutellar extract from 2-hr imbibed seed. Purified ADH was prepared as previously described (3).

**Density Gradient Centrifugation.** The procedure was essentially as previously described (4, 8). Each 4-ml nitrocellulose tube contained 2 ml of saturated CsCl with NAD (3.5 mg/ml), 2 ml of crude enzyme extract, and 20 μg of lactate dehydrogenase (E.C. 1.1.1.27; Sigma Chem. Co.) as a marker, all

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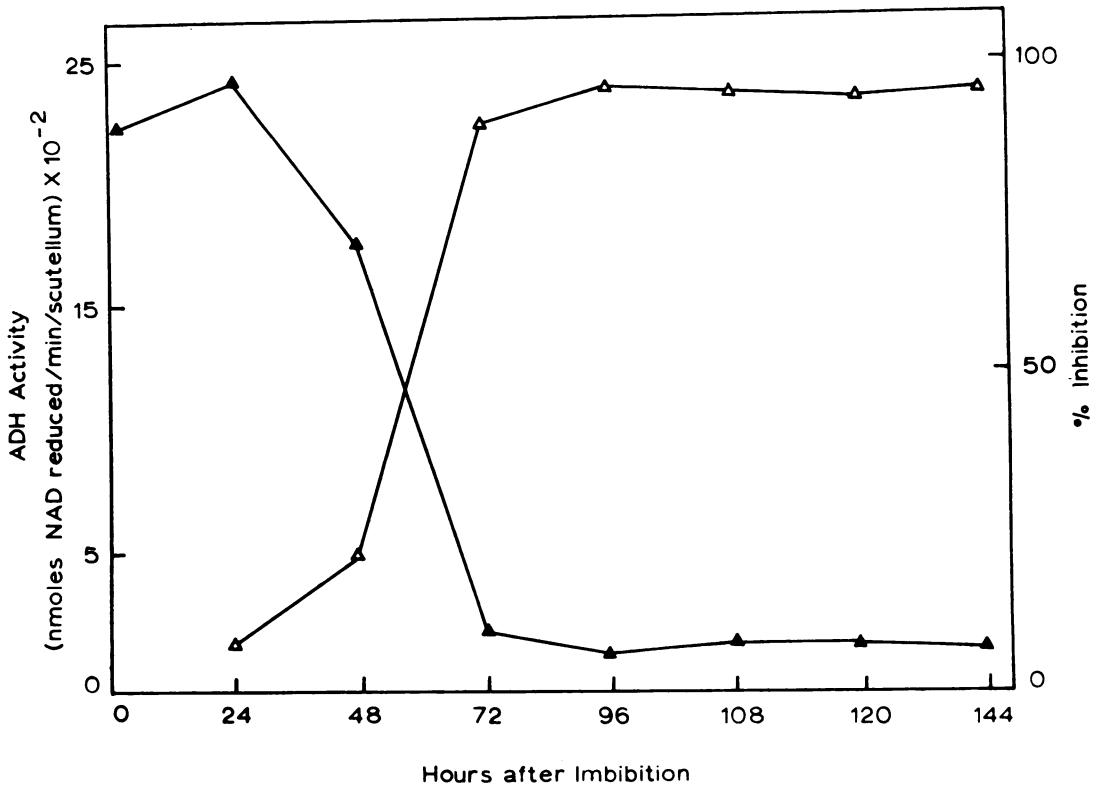


FIG. 1. Developmental time course of alcohol dehydrogenase activity and its specific inhibitor in maize scutella during seed germination ADH activity (▲); inhibitor activity (△) is the per cent inhibition of ADH by reciprocal mixing of scutellar extracts from 2-hr imbibed seeds and later developmental stages. Mixtures were incubated at 25 C for 3 hr.

uniformly mixed. The tubes were centrifuged at 17,000 rpm for 65 hr at 3 C in a Beckman L-3 ultracentrifuge equipped with an SW-65 rotor. After centrifugation, the tubes were punctured with a No. 22 needle and 3-drop fractions were collected in the cold. The refractive index of every 10th fraction was determined with a Bausch & Lomb Abbe-32 refractometer and converted to density units. Enzyme activities were measured soon after puncturing the tubes. Gaussian plots were used to determine the density and the half-height width of the enzyme activity peaks.

## RESULTS AND DISCUSSION

The activity of alcohol dehydrogenase in the scutellum remains at a high level for the first 24 hr after germination. After that, the activity decreases sharply (Fig. 1). The developmental pattern of alcohol dehydrogenase activity in the excised cultured scutella was similar to that in intact tissue. Neither 10 μg/ml cycloheximide nor 50 μg/ml actinomycin D had any significant inhibitory effect on alcohol dehydrogenase activity (Table I). In contrast 2 μg/ml cycloheximide and 50 μg/ml actinomycin D inhibit the increase of malate dehydrogenase activity in maize scutella completely in a short period of incubation (15) indicating that the insensitivity of ADH development to these inhibitors cannot be due to general ineffectiveness of these inhibitors (*e.g.* because of lack of penetration) in the maize scutellum. Therefore, our data indicate that ADH molecules are not synthesized during seed germination.

In order to confirm the above suggestion, we carried out the density labeling experiment. After careful determination of the position of the ADH activity peak in CsCl density gradients by Gaussian plot (Figs. 2 and 3), we found that ADH molecules from scutella of seeds germinated in the presence of

Table I. *Effect of Cycloheximide and Actinomycin D on Development of Maize Scutellar Alcohol Dehydrogenase during Seed Germination*

| Treatment | Hr after Start of Water Imbibition | | |
|---|---|---|---|
| | 24 | 72 | 168 |
| | *ADH activity (nmoles NAD reduced/scutellum·min) × 10⁻²* | | |
| Intact scutellum | 23.4 | 11.5 | 0.8 |
| Excised scutellum grown in nutrient[1] | 23.4 | 13.5 | 2.5 |
| Excised scutellum grown in nutrient with cycloheximide | 23.4 | 14.4 | 2.0 |
| Excised scutellum grown in nutrient with actinomycin D | 23.4 | 14.0 | 0.75 |

[1] Scutellum was excised from 24-hr-old seedling and cultured in Hoagland's solution.

$^{15}NH_4Cl$ and $D_2O$ for 36 hr had exactly the same apparent buoyant density ($\rho = 1.297$) as the ADH molecules from scutella of seeds germinated in $^{14}NH_4Cl$ and $H_2O$. The half-height widths of the ADH activity peak in both samples were identical (42 drops; Figs. 2 and 3). Thus, no measurable amount of heavy isotopes was incorporated into the ADH molecules within the first 36 hr of germination. Catalase in scutella of the same inbred (W64A) and at the same developmental period showed a density shift as large as 0.019 g/ml when seeds were germinated in the presence of $K^{15}NO_3$ and $D_2O$ under similar conditions as described above (8). Thus the unchanged buoyant density of ADH molecules is not due to the failure to replace H by D, or $^{14}N$ by $^{15}N$ in the amino acid

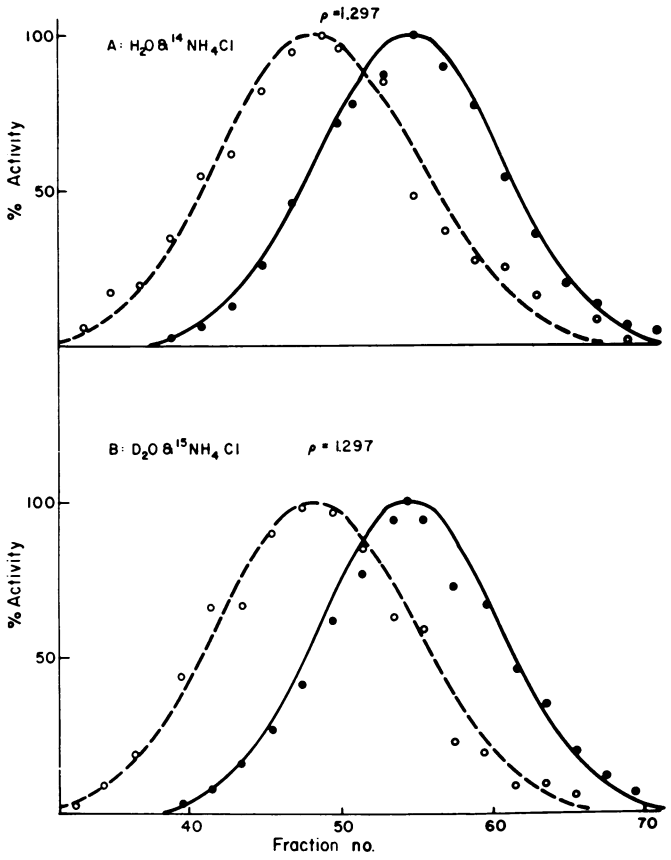


FIG. 2. Equilibrium distribution in CsCl gradients of scutellar extract ADH from seeds germinated for 36 hr on either (A) $^{14}NH_4Cl$ in $H_2O$ (control) or (B) 10 mM $^{15}NH_4Cl$ in 70% $D_2O$. Activity of lactate dehydrogenase (marker) (●——●); activity of alcohol dehydrogenase (○---○). Note absence of any density shift.

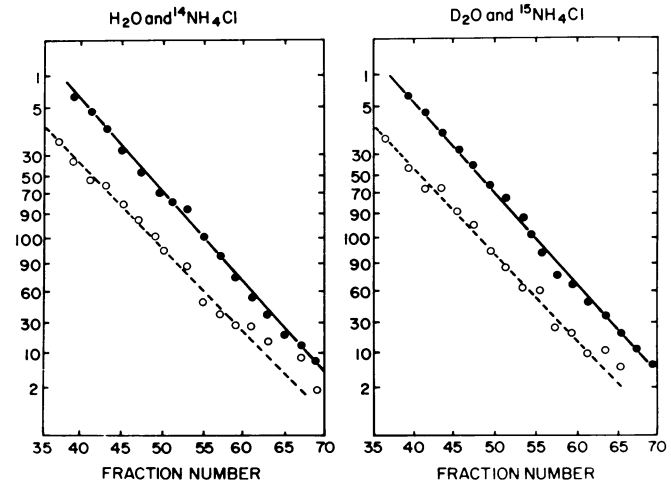


FIG. 3. Gaussian plot of the points on the curves in Figure 2. LDH activity (●——●); ADH activity (○---○).

molecules. Four different alcohol dehydrogenase isozymes are present in the maize scutellum after germination (ADH-1, ADH-2, ADH-3, and ADH-4); ADH-2, the most abundant isozyme, contributes more than 95% of the total ADH activity. Test tube assays cannot distinguish individual isozymes in a mixture, yet there should be a density shift of the enzyme activity peak due to the incorporation of heavy isotopes into any one (or more) of the isozymes, or the width of the activity peak would be broadened if only a minor isozyme were synthesized *de novo*. Although other possibilities controlling the formation of a functional protein have not been ruled out, our results indicate that the ADH genes were repressed either before or at the onset of the germination process.

Inhibitors of nitrate reductase (12, 13), invertase (7, 14), and proteases (9) have been found in higher plants. This result suggests that inhibition of enzyme activity may be an important mechanism of enzyme regulation in higher plants. In maize, the presence of a "two-factor" ADH inhibitor had been reported previously (2). According to that theory, one "factor" is generated from the embryo and another from the root; it was proposed that ADH is inhibited when both "factors" come together (2). The alcohol dehydrogenase inhibitor we describe here is specific for ADH, and pure or crude maize ADH preparations are effectively inhibited by endogenous ADH inhibitor isolated from root, shoot, or leaf alone (Table II); malate dehydrogenase and other dehydrogenases present in the same tissue and developmental stages (6) were not inhibited by this ADH inhibitor. The ADH inhibitor activity is present in the scutellum, shoot, leaf, and root of the seedling. The inhibitor is heat-labile but not sensitive to trypsin treatment and is partially dialyzable. β-Mercaptoethanol protects ADH activity against the inhibitor, while ethanol, or $NAD^+$ has little protective effect against the inhibitor (Table III). There is no effect of β-mercaptoethanol on endogenous scutellar protease activity as determined by use of azocasein. One interpretation of this result is that the ADH inhibition is not due to nonspecific degradation by protease. An alternate interpretation is that the "inhibitor" is in fact a protease, and that the β-mercaptoethanol in some way specifically protects the ADH molecule from degradation. We believe the former hypothesis to be more likely, and are presently attempting to resolve the question. As shown in Figure 1, the inhibitor level, which can be assayed by reciprocal mixing of scutellar extracts from different stages of germination, was very low before 40 hr of germination. However, it increased at the same time when ADH activity started to decline. ADH

Table II. *Tissue Distribution of ADH Inhibitor in Maize*

β-Mercaptoethanol (100 mM) and NAD (350 μg/ml) was used. Scutellar extract was prepared from 3-hr imbibed seeds. Root, shoot, and leaf extracts were prepared from 8-day-old seedlings.

| Treatment | Residual ADH Activity | Inhibition |
|---|---|---|
| | % | |
| Scutellar extract | 100 | 0 |
| SE + root extract | 45 | 55 |
| SE + RE + SH[1] | 91 | 9 |
| SE + RE + NAD | 64 | 37 |
| SE + leaf extract | 65 | 35 |
| SE + LE + SH | 88 | 12 |
| SE + LE + NAD | 85 | 15 |
| SE + shoot extract | 48 | 52 |
| SE + ShE + SH | 85 | 15 |
| SE + ShE + NAD | 90 | 10 |

[1] SE: scutellar extract; RE: root extract; SH: β-mercaptoethanol; LE: leaf extract; ShE: shoot extract.

Table III. *Properties of ADH Inhibitor in Maize Scutellum*
Enzyme represents scutellar extract from 2-hr imbibed seed. Inhibitor represents scutellar extract from 8-day-old seedling; no detectable ADH activity was found in this preparation.

| Treatment | Residual ADH Activity |
|---|---|
| | % |
| Experiment 1 | |
| Enzyme only | 100 |
| Enzyme + inhibitor | 9 |
| Enzyme + inhibitor + β-SH[1] (50 mM) | 81 |
| Enzyme + inhibitor + β-SH (10 mM) | 63 |
| Enzyme + inhibitor + DTT (3.5 mM) | 33 |
| Enzyme + inhibitor + NAD (0.14 mg/ml) | 25 |
| Enzyme + inhibitor + Ethyl alcohol (0.5%) | 16 |
| Experiment 2 | |
| Enzyme only | 100 |
| Enzyme + inhibitor | 14 |
| Enzyme + inhibitor (boiled) | 84 |

[1] β-SH: β-mercaptoethanol; DTT: dithiothreitol.

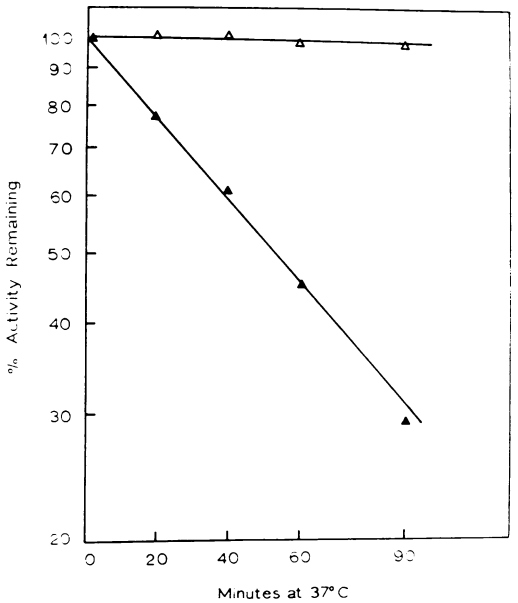


FIG. 4. Stability of alcohol dehydrogenase activity at 37 C. ADH activity from 2-hr imbibed seed (△——△); ADH activity from 4-day-old seedlings (▲——▲). Enzyme was extracted from scutella.

activity in scutellar extracts from different stages of germination also decreases with time without reciprocal mixing if the extracts are incubated at 37 C. This activity is probably also due to the presence of the ADH inhibitor, since the extent of ADH activity decrease is greater for the scutellar extracts from later stages of germination (Fig. 4). Excised scutella cultured on nutrient media had even higher inhibitor activity than a comparable extract from scutella from intact seedlings (99.5% and 86% inhibition, respectively) indicating that the inhibitor can be synthesized in the scutellum and is not merely transported to this organ from other parts of the seedling.

The increase of the inhibitor level in the scutellum is concomitant with the decline of ADH activity after seed germination. This fact, accompanied with the results from the metabolic inhibitor experiments, led us to conclude that the control of the decline in ADH activity after germination does not result from reduced levels of new synthesis of ADH molecules, but results from the build up of a specific inhibitor to the enzyme. Experiments to purify the ADH inhibitor by affinity chromatography for further characterization are now underway.

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